

Protective Effect of *Withania somnifera* and *Cardiospermum halicacabum* Extracts Against Collagenolytic Degradation of Collagen

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Received: 29 December 2010 / Accepted: 7 July 2011 /
Published online: 26 July 2011
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Abstract The irreversible destruction of extracellular matrix (ECM) such as cartilage, tendon, and bone that comprise synovial joints is the hallmark of both rheumatoid arthritis and osteoarthritis by over-expression of matrix metalloproteinase (MMP)-collagenases. We report herein the detailed study on the inhibitory effects of *Withania somnifera* extract (WSE) and *Cardiospermum halicacabum* extract (CHE) on *Clostridium histolyticum* collagenase (ChC) activity against the degradation of the ECM component of bovine Achilles tendon type I collagen by hydroxyproline assay method. Interaction of WSE and CHE with ChC exhibited 71% and 88% inhibition, respectively, to the collagenolytic activity of ChC against collagen degradation, and the inhibition was found to be concentration-dependent. The inhibition kinetics of ChC by both the extracts has been deduced from the extent of hydrolysis of *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala. Both WSE and CHE are provided competitive and mixed type inhibition on ChC activity, respectively. Circular dichroism studies of ChC on treatment with WSE and CHE revealed changes in the secondary structure of collagenase. These results suggest that the WSE and CHE facilitated collagen stabilization through collagenase inhibition.

Keywords Extracellular matrix · Matrix metalloproteinases · Collagen · Collagenase · Collagenase inhibition kinetics

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Introduction

Controlled synthesis and degradation of collagen is a balanced feature in a variety of physiological conditions such as embryonic development, tissue remodeling, tissue repair, etc. In pathophysiological conditions, uncontrolled breakdown and synthesis of collagen lead to arthritis, invasion and metastasis of malignant tumors, atherosclerosis, fibrosis, aneurysm, etc. The matrix metalloproteinases (MMPs) is a large family of calcium-dependent zinc-containing endopeptidases (also called matrixins), which are involved in and responsible for the remodeling and degradation of extracellular matrix (ECM) including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycans. The MMPs are synthesized and released by most of the normal and abnormal cells, including fibroblasts, macrophages, neutrophils, osteoclasts, etc. [1–3].

Over-expression of MMPs also plays a role in pathological conditions involving untimely and accelerated turnover of ECM, including rheumatoid arthritis (RA) and osteoarthritis (OA), and invasion and metastasis of malignant tumors. The rheumatoid tissue produces the excess amount of collagenases, and it destroys the articular cartilage and invades the autologous joint. Possible involvement of chondrocytes in destruction of cartilage in OA has been suggested by the finding of collagenase. Wounding of skin was also shown to induce collagenase formation by both epithelial and mesenchymal cells at the wound margin [4–6].

MMP-1, 8, and 13 (collagenases I, II, and III) and MMP-18 are capable of degrading native interstitial collagens I, II, III, IV, and X at a specific site between Gly775–Ile776 of the $\alpha 1$ chains and Gly775–Leu776 residues of $\alpha 2$ chains at the $\frac{3}{4}$ length from the N terminus of the molecule [7, 8]. MMP-1 is expressed by various normal cells, for example, fibroblast, keratinocytes, endothelial cells, macrophages, chondrocytes, and osteoblasts, as well as by many different types of tumor cells. Human MMP-8 is expressed by chondrocytes, rheumatoid synovial fibroblasts, gingival fibroblasts, oral mucosa keratinocytes, and melanoma cells. MMP-13 expression is also detected in pathological conditions that are characterized by the destruction of normal collagenous tissue, e.g., in OA cartilage, rheumatoid synovium, atherosclerosis, and aortic aneurysms, etc. MMPs have recently become interesting targets for drug design in the search for novel types of anti-cancer, anti-arthritic, and other pharmacological agents. These MMP inhibitors are useful in the management of osteoporosis, aortic aneurysm, and multiple sclerosis, among others. Several synthetic MMP inhibitors have been tested in clinical trials evaluating their ability to control the arthritis and invasion of malignant tumors in vivo [4, 5, 8]. However, limited performance of MMP inhibitors in phase III clinical trials were disappointing. Therefore, using the broad spectrum of MMP inhibitors selectively targeting certain MMPs may be preferable. Novel inhibition strategies for MMPs may serve as important approaches for targeted therapy for treatment of pathological conditions [9, 10].

Many pharmacological studies have been carried out to describe multiple biological properties of *Withania somnifera* root, leaf, and fruit extracts, one of the most widely used herbs in Ayurvedic medicine named Ashwaghandha. This plant extract contains several alkaloids, withanolides, a few flavanoids, and reducing sugars, and is a rich source of bioactive compounds [11, 12]. This extract possessed antioxidant, anti-microbial, anti-enzymatic, effective protein antigenicity reduction, anti-mutagenic, and anti-carcinogenic properties. This extract has been linked with inhibitory and preventive effects in various human cancers and cardiovascular diseases. The chondroprotective potential of root extracts of *W. somnifera* in OA have been studied [12–15]. This extract could have the therapeutic role in the prevention of glycation-induced pathogenesis in diabetes mellitus and aging [16].

Cardiospermum halicacabum, family Sapindaceae, is a deciduous, branching, herbaceous climber. This is distributed throughout the plains of India and used in the treatment of arthritis, chronic rheumatism, and stiffness of limbs, and its root is used for nervous diseases, as a

diaphoretic, diuretic, laxative, refrigerant, etc. [17]. This plant extract contains flavones, aglycones, triterpenoids, and glycosides, and a range of fatty acids and volatile ester has been reported [18, 19]. Although scientific evidence is mounting to support the claims that WSE and CHE may be a remedy for OA and RA, it is still limited to traditional rural cultures.

The inhibition of MMP-1, MMP-8, and MMP-13 by plant extracts such as flavonoids and polyphenolic was confirmed by gelatin zymography and was observed for MMPs associated with various connective tissues. Plant extracts were tested for their ability to inhibit the prokaryotic and eukaryotic cell-derived collagenase activities. Pre-incubation of bacterial collagenases with flavonoid and polyphenol reduced the collagenolytic activity as well [20].

In addition to mammalian MMPs, there are also some other enzymes such as bacterial collagenases, which can degrade ECM. One such bacterial collagenase known as *Clostridium histolyticum* collagenase (ChC), which belongs to the family of M-31 metalloproteinase family, is capable of hydrolyzing triple-helical region of collagen under physiological conditions, as well as many synthetic peptides. In fact, the crude homogenate of ChC, which contains several distinct collagenase isozymes, is the most efficient system known for the degradation of ECM. MMPs-collagenases and ChC, though relatively different, are considered to act through the same mechanism of action in the hydrolysis of collagens and synthetic substrates [21–24]. Similar compositions of amino acids are present in the active site of mammalian and bacterial collagenases. Moreover, commercially available bacterial collagenases represent a mixture of various iso-enzymes with different activities.

In this study, we evaluated the protective effect of collagen by WSE and CHE against collagenase degradation

Materials and Methods

Materials

All reagents and chemicals used were of analytical grade. Collagenase (type IA) and *N*-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) were sourced from Sigma Chemicals Co., USA. All other reagents and chemicals used for the study were sourced from SRL Ltd., India.

Preparation and Purification of WSE and CHE

Commercially available Ayurvedic powders of *W. somnifera* and *C. haliacabum* were percolated four times with ethanol/water (1:3) at room temperature [13]. The combined extracts were filtered, centrifuged, and concentrated to one sixth of the original volume under reduced pressure at a temperature of 50 ± 5 °C. Finally, the extract was completely dried under vacuum in the desiccator. Total yield of the WSE and CHE were 15% and 17%, respectively. For the isolation of flavonoids, steroidal glycosides, and withanolides/glucowithanolides, the aqueous ethanolic extract (1:3) was dissolved in water, and the solution was successively extracted with chloroform in a separating funnel. Both the chloroform fractions were separately concentrated under reduced pressure to yield the residues containing flavonoids, steroidal glycosides, and withanolides/glycowithanolides.

Preparation of BAT

Tendons from bovine Achilles legs were collected and washed with 0.9% NaCl at 4 °C to remove the adhering muscles and other soluble proteins. The bovine Achilles tendon (BAT)

was subsequently washed extensively in double-distilled water and treated with 0, 5, 10, 20, 40, and 80 μg WSE and CHE for 24 h at room temperature (27 °C) without any agitation. After 24 h, both the extracts treated with BAT were stored in water at 4 °C before testing for resistance to ChC.

ChC Hydrolysis of WSE- and CHE-Treated BAT

Percentage (%) of collagen degradation was calculated by the following method [25]. The native, WSE- and CHE-treated BAT were further treated with ChC. ChC treatment was carried out in 0.04 M CaCl_2 solution buffered at pH 7.2 with 0.05 M Tris–HCl. The collagen/ChC ratio (25 mg BAT, 0.5 mg ChC) was maintained at 50:1. The samples were incubated at a temperature of 37 °C. Samples were collected at various time intervals ranging from 6 to 96 h and stored in freezer. The cleavages of native and treated BAT were monitored by the release of soluble form of hydroxyproline from insoluble collagen [26]. Aliquots of 750 μl of supernatant were withdrawn after centrifuging at 10,000 rpm for 10 min. The ChC hydrolysates were hydrolyzed with 6 N HCl in sealed hydrolysis tubes for 16 h. The hydrolysates were evaporated to dryness in a porcelain dish over a water bath to remove excess acid. The residues of free acid were made up to a known volume, and the percentage of hydroxyproline was determined using the method by Ryan and Woessner [24]. Hydroxyproline is a unique amino acid for collagen, and it offers itself as a useful marker for identifying collagen in the presence of non-collagenous proteins. The method of determining hydroxyproline involves the oxidation of hydroxyproline to pyrrole-2-carboxylic acid, which complexes with *p*-dimethylaminobenzaldehyde exhibiting maximum absorbance at 557 nm.

The % soluble collagen = % hydroxyproline \times 7.4.

Based on the soluble (solubilized due to enzymatic hydrolysis) collagen content in the supernatant solution of the ChC-treated BAT, the percent degradation of collagen for native, WSE-, and CHE-treated tendons are calculated as

$$\% \text{ Collagen degradation} = 100 - \left(\left(\frac{\text{Initial collagen} - \text{Soluble collagen}}{\text{Initial collagen}} \right) \times 100 \right)$$

Monitoring of BAT Degradation by Inhibition of ChC Activity

Percent inhibition of ChC by WSE and CHE was calculated by the following method [25]. A known amount of ChC in 1 ml of 0.1 M Tris–HCl (pH 7.4) containing 0.05 M CaCl_2 at 25 °C was incubated with 0, 5, 10, 20, 40, and 80 μg of WSE and CHE for 18 h. Subsequently, the BAT was treated with the incubated samples of native, WSE-, and CHE-treated ChC. The ratio of collagen/ChC was maintained at 50:1 and the reaction buffered at pH 7.4 using 0.1 M Tris–HCl and 0.05 M CaCl_2 . The treated samples were incubated at 37 °C, and after 72 h, the reaction was stopped, and the mixture was centrifuged for 15 min at 10,000 rpm. The supernatant was analyzed for hydroxyproline [26], and percent collagen degradation was determined. Percent inhibition was calculated as differences in the percent degradation of BAT collagen treated by native, WSE-, and CHE-treated ChC.

$$\% \text{ Inhibition} = \left(\frac{\% \text{ Collagen degradation}_{\text{native ChC}} - \% \text{ Collagen degradation}_{\text{plant extract}}}{\% \text{ collagen degradation}_{\text{native ChC}}} \times 100 \right)$$

Extraction of BAT Collagen

The BAT, collected fresh from local slaughter house, were manually dissected out from surrounding fascia, followed by washing in distilled water. They were cut into small bits of 3–4 mm each with a sharp knife and were solubilized by a patented procedure [27].

Inhibition of ChC Activity by Zymography Analysis

The ChC is treated with varying concentrations viz., 0, 5, 10, 20, 40, and 80 μg of aqueous solution of WSE and CHE for 24 h at 25 °C. Both the extract-treated ChCs were directly applied to a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) that contained 0.1% collagen. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 30 min, three times with water for 10 min, and incubated in a solution of 50 mmol/l Tris–HCl, 5 mmol/l CaCl₂, 1 $\mu\text{mol/l}$ ZnSO₄ (pH 8.0) for 5 days at 37 °C. The gel was stained with Coomassie blue.

Kinetic Investigations on the Assay of Native ChC

ChC assay using FALGPA as the substrate was performed according to the method reported earlier [28]. Assays were carried out spectrophotometrically by continuously monitoring the decrease in absorbance of FALGPA after the addition of ChC. FALGPA (at concentrations of 0.1–1.6 mM) was taken in the appropriate amount of Tricine buffer (0.05 M Tricine, 0.4 M NaCl and 10 mM CaCl₂, pH 7.5) and ChC (100 μl of 0.4 mg/ml) was added, and the final volume was adjusted to 1 mL. The course of hydrolysis of FALGPA was monitored using Varian Cary 100 UV–visible spectrophotometer by following the decrease in absorbance at 324 nm when [FALGPA]=0.1 mM. At higher concentrations of FALGPA viz. (0.2 mM) and (0.4–1.6 mM), the decrease in absorbance was measured at 338 and 345 nm, respectively. An initial rate treatment was adopted by treating the first 10% of hydrolysis according to standard methods [29].

Kinetic Investigations on Inhibition of ChC

The reaction of WSE- and CHE-treated ChCs with FALGPA were performed under the same conditions mentioned as employed for the assay of native ChC in this study. The ChC is treated with varying concentrations viz., 0, 10, 20, 40, 80, and 160 μg of aqueous solution of WSE and CHE for 18 h at 25 °C. The final concentrations of the ChC in all the treatments are maintained constant (0.4 mg/mL). The FALGPA at concentrations of 0.1–1.6 mM was taken in appropriate amount of Tricine buffer (0.05 M Tricine, 0.4 M NaCl, and 10 mM CaCl₂, pH 7.5); WSE-incubated ChC (100 μl) was added, and the final volume was adjusted to 1 mL. The hydrolysis of substrate was monitored at the corresponding wavelengths (immediately after the addition of both the extract-incubated ChCs) as done in the case of native ChC. The concentrations of substrate (FALGPA) used were in the range of 0.1–1.6 mM. Rates of hydrolysis were calculated employing initial rate methods. The rate data were analyzed in terms of Michaelis–Menten treatment. From the Lineweaver–Burk plots of v^{-1} vs $[S]^{-1}$, the kinetic parameters such as V_{max} , maximum velocity, and K_m and the Michaelis–Menten constant of the enzyme were calculated. Initial velocities were calculated from the slope of the absorbance changes during the first 10% of hydrolysis and converted into units of microkatal (micromoles per second) by dividing to full hydrolysis and multiplying by the substrate concentration.

Circular Dichroism Spectral Analysis of WSE- and CHE-treated ChCs

Circular dichroism spectrum of type IA ChC of concentration 0.18 mg/ml in 1 mM acetate buffer (pH 4.0) was acquired at 25 °C using Jasco-J715 Spectropolarimeter. The conformational changes in ChC were investigated after incubating the enzyme with varying concentrations (10–80 µg) of WSE and CHE.

Statistical Analysis

Results are represented as mean±SD of at least three experiments. A probability level of $P < 0.001$ was considered statistically significant, and the analysis was done by Student's *t* test. Statistically significant differences between groups was ascribed at $**P < 0.001$ and $*P < 0.01$.

Results

We have carried out a study to determine the protective potential of WSE and CHE on collagenolytic activity. It is of profound interest to establish how the WSE and CHE exhibit dose-dependent inhibition on ChC activity against the degradation of collagen.

Stability of WSE- and CHE-treated Collagen Against ChC Activity

The percent of collagen degradation (based on hydroxyproline released) for the native, WSE- and CHE-treated BAT by ChC at various time periods has been determined (Fig. 1a, b). Significant reduction in the degradation of collagen was observed for the BAT treated with the WSE and CHE compared with native BAT. WSE-treated BAT exhibited 37.64% (at 80) degradation at 72 h period of incubation, whereas CHE-treated BAT exhibited only 33.35% degradation for the same duration of incubation. WSE and CHE can interact with collagen through hydrogen bonding and hydrophobic interactions. The hydrophobic core of the steroidal lactones incorporates itself likely into hydrophobic areas, while hydroxyl moieties of steroids may establish multiple hydrogen bonds with neighboring collagen molecules, resulting in improved stability and preventing the free access of ChC to active sites on the collagen chains. The stability of WSE- and CHE-treated collagen against ChC would have been brought about by protecting the active sites. The significant differences in the enzymatic stability offered by WSE and CHE could be due to the effectiveness of the latter in exhibiting better interaction with collagen through multiple hydrogen-bonded crosslinks. It is important to probe further the ability of WSE and CHE in the direct inhibition of ChC.

Inhibition of ChC by WSE and CHE Against BAT Collagen Degradation

Inhibition of ChC activity was tested using BAT collagen as a substrate. WSE- and CHE-treated ChC exhibited the dose-dependent inhibition on the collagenolytic activity against collagen degradation (Fig. 2a, b). The inhibition increased with the increase in concentration of WSE and CHE at 80 µg concentration; 76.66% and 86.66% inhibition have been observed against ChC hydrolysis at 72 h. WSE and CHE having the unit of steroidal lactone, glycoside, and its hydroxyl groups can exhibit better hydrogen bonding and hydrophobic interactions with ChC and hence can influence significant changes in the conformation resulting in high inhibition of ChC activity against collagen degradation.

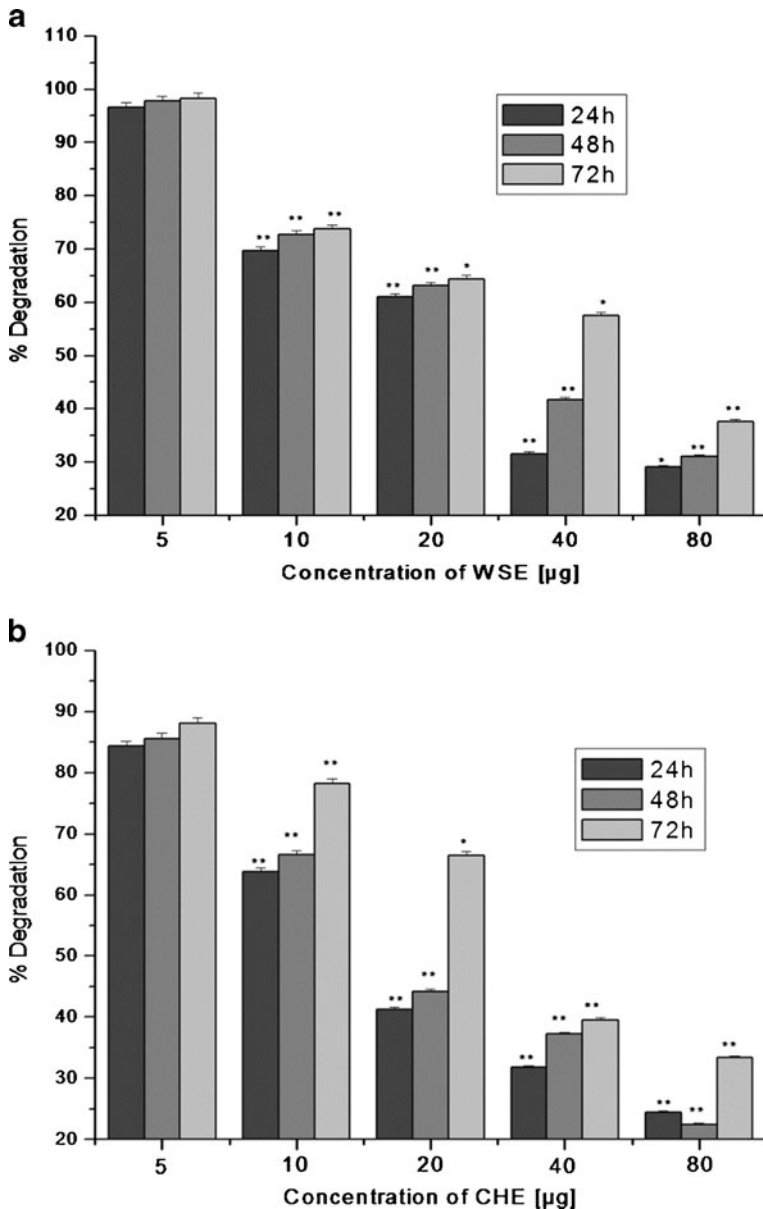


Fig. 1 The percent of degradation of **a** WSE- and **b** CHE-treated BAT collagen by ChC. **Statistically significant differences between groups ($P < 0.001$). *Statistically significant differences between groups ($P < 0.01$)

Effect of WSE and CHE on ChC Activity by Collagen Zymography

This study was conducted by isolating BAT collagen. Purity of collagen was confirmed by SDS-PAGE analysis (Fig. 3a). The ChC inhibition assay of WSE- and CHE-treated ChC was determined by collagen zymography. The collagen degradation by native ChC was shown as

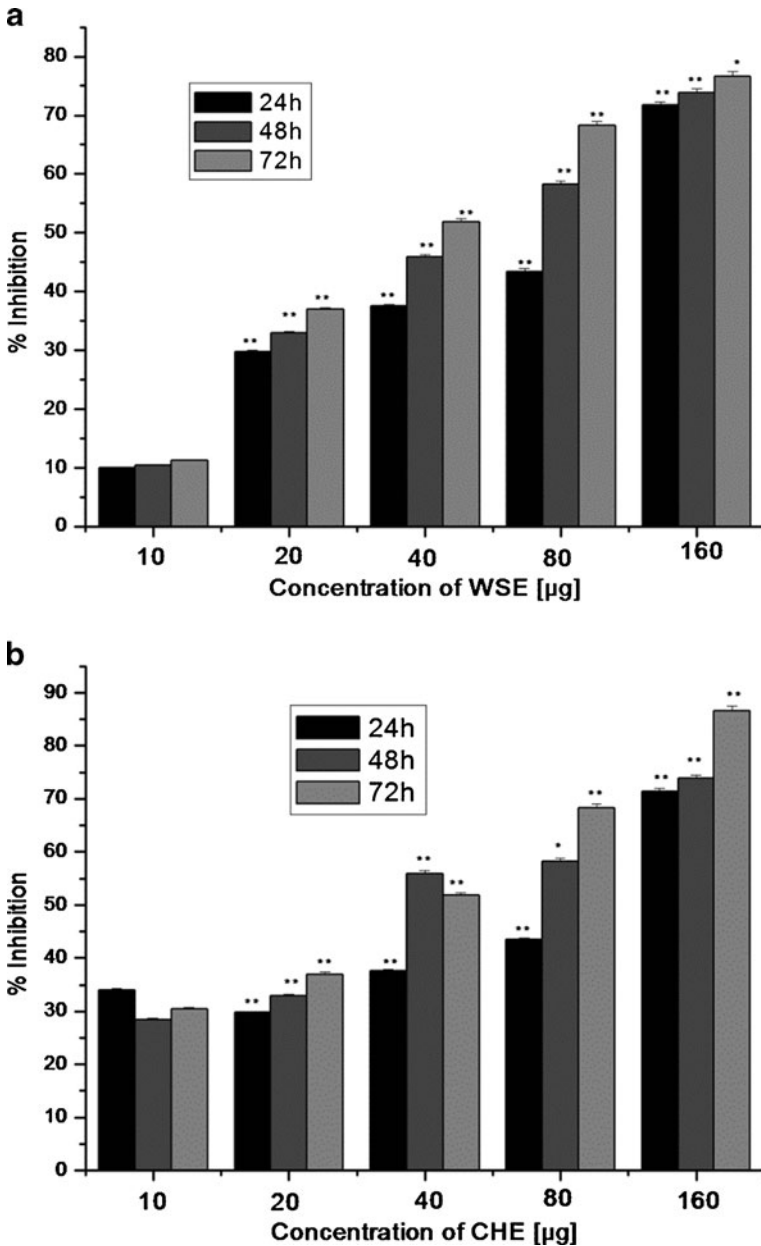
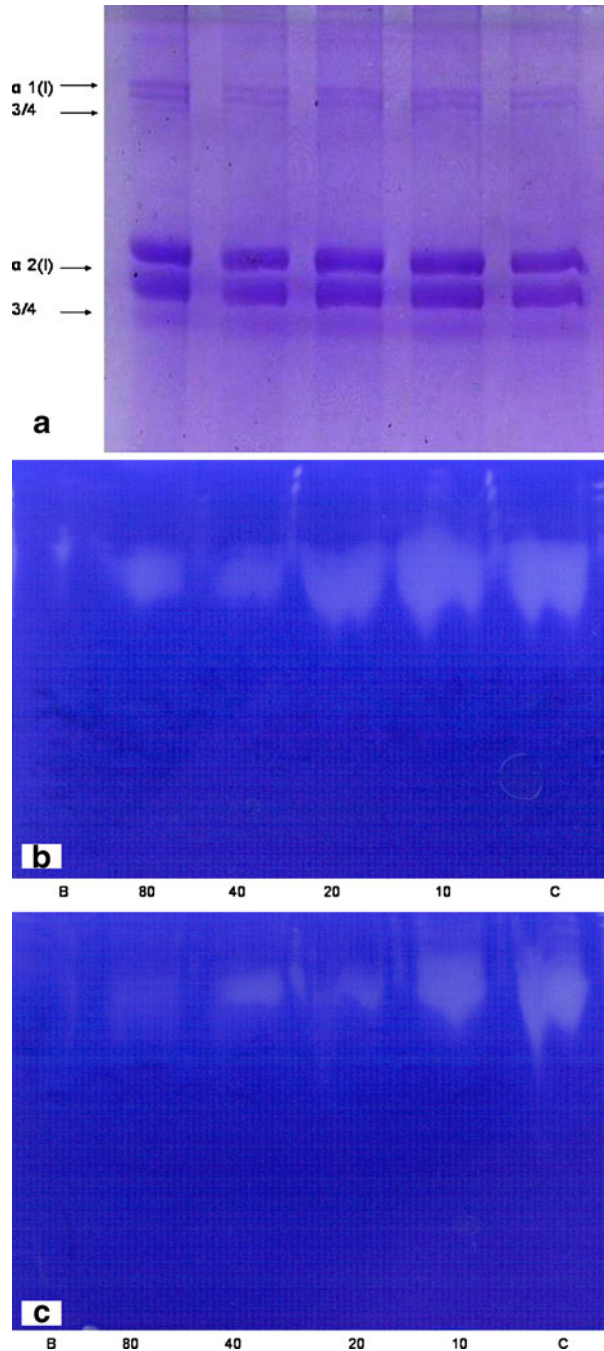


Fig. 2 The percent of inhibition of ChC by **a** WSE and **b** CHE at various concentrations. **Statistically significant differences between groups ($P < 0.001$). *Statistically significant differences between groups ($P < 0.01$)

the clear area, and the intensities of the area were quantified by densitometric scanning and expressed in relative intensity to collagen as a substrate. Considering clear band intensity of degraded collagen by native ChC as 100 after incubation for 24 h, WSE- and CHE-treated ChC had much fewer clear bands. Both the extract-treated ChCs had high inhibition (90%)

Fig. 3 SDS-PAGE of **a** BAT collagen, and gelatin zymography of **b** WSE- and **c** CHE-treated ChCs



against collagen degradation. WSE and CHE exhibited a dose-dependent inhibitory effect on ChC activity (20% at 10 $\mu\text{g/ml}$, 60% at 40 $\mu\text{g/ml}$, and 75% at 80 $\mu\text{g/ml}$), and complete inhibition was observed at CHE concentrations above 80 $\mu\text{g/ml}$ (Fig. 3b, c).

Kinetic Analysis of the Inhibition of ChC

In order to establish the mechanism of inhibition of ChC activity by WSE and CHE, their ability to hydrolyze of FALGPA has been studied. The data were presented in a Lineweaver–Burk plot, i.e., double-reciprocal plot and Dixon plot to determine the kinetic parameters (Figs. 4a, b and 5a, b). The kinetic parameters like K_m , V_{max} , and K_i were calculated and tabulated (Table 1).

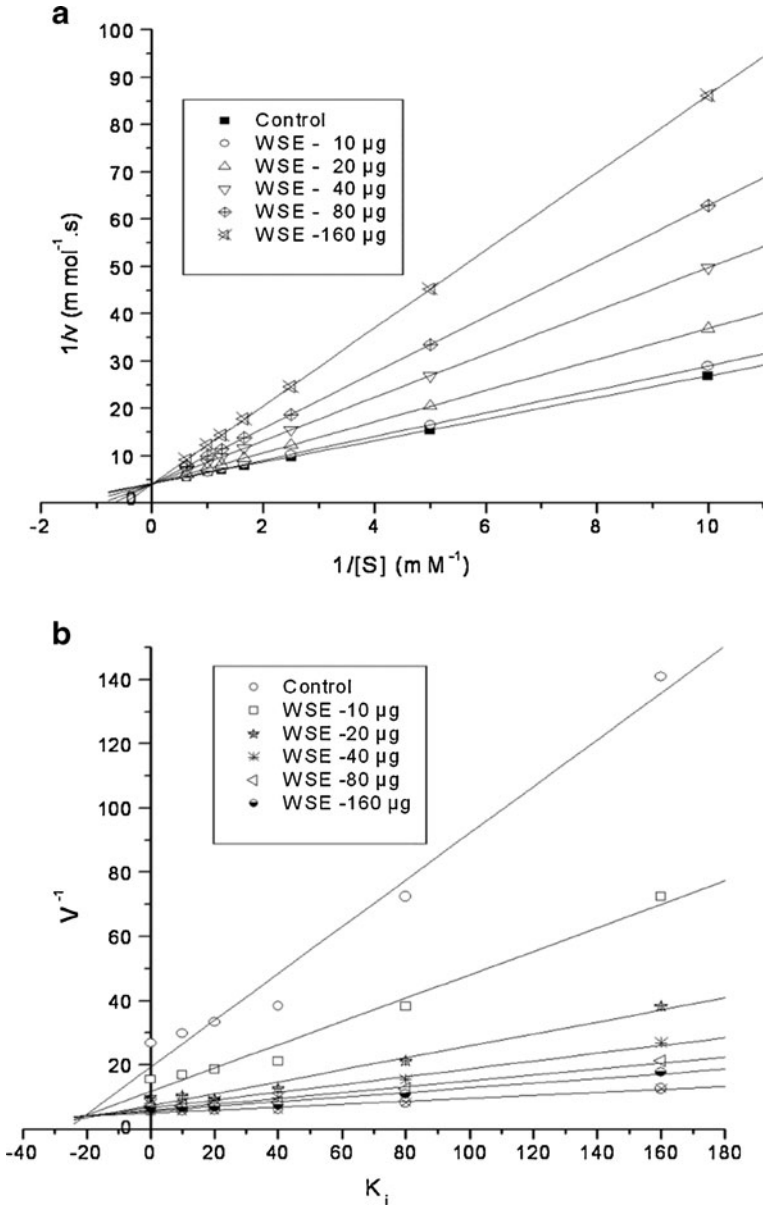


Fig. 4 Lineweaver–Burk of FALGPA hydrolysis by ChC in the presence of **a** WSE and **b** CHE. Assays were performed in Tricine buffer, pH 7.5, with varying concentrations of FALGPA (0.1–1.6 mM)

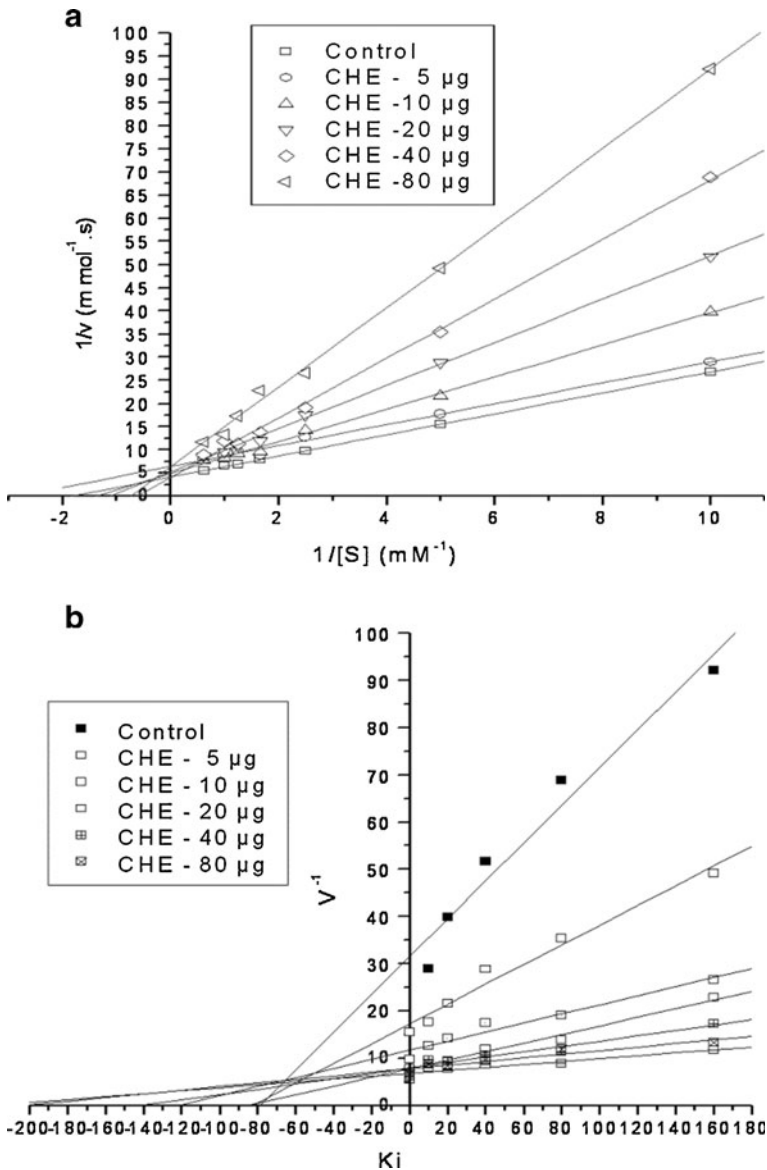


Fig. 5 Dixon plots of FALGPA hydrolysis by ChC in the presence of **a** WSE and **b** CHE respectively. Assays were performed in Tricine buffer, pH 7.5, with varying concentrations of FALGPA (0.1–1.6 mM)

Inhibition Kinetics of ChC

To determine the nature of the inhibition of ChC, experiments were carried out using the known amount of ChC in the presence or absence of various concentrations of WSE and CHE. The velocity (v) of the reaction was calculated for each substrate concentration (S), and the data were presented in a Lineweaver–Burk plot ($1/v$ versus $1/S$) to determine the kinetic parameters. The Lineweaver–Burk plot obtained for FALGPA hydrolysis by ChC

Table 1 Michaelis–Menten parameters for ChC hydrolysis of FALGPA at 25 °C, pH 7.5 in the presence of varying concentrations of WSE and CHE

Nature of treatment	Plant extract [μg]	K_m (mM)	V_{\max} (mmol s^{-1})	Type of inhibition	K_i (μg)
Control	–	0.555	0.2439		
	10	0.625			
	20	0.714			
WSE	40	0.833	0.2439	Competitive type	20
	80	1.666			
	160	3.333			
	10	0.349	0.1551		
	20	0.709	0.2059		
CHE	40	0.893	0.1902	Mixed type	
	80	1.351	0.2418		
	160	1.612	0.1598		

incubated with different concentrations of WSE and CHE are shown (Figs. 4a and 5a). The Lineweaver–Burk plots, i.e., double-reciprocal plots obtained for FALGPA hydrolysis by ChC incubated in the presence of WSE revealed that there was no change in the $1/V_{\max}$ compared with control. We know that V_{\max} is unchanged by the presence of a competitive inhibitor, and CHE revealed that there was the change in the $1/V_{\max}$ by the presence of a mixed type of inhibitor. Increasing concentration of an inhibitor [I] results in a family of lines with different slopes because the intercept on the $1/V_0$ axis is equal to $1/V_{\max}$. That is, regardless of the concentration of a competitive inhibitor, a sufficiently high substrate concentration will always displace the inhibitor from the enzyme active site. WSE and CHE exhibited dose-dependent inhibition on ChC activity, and Lineweaver–Burk plots clearly showed a competitive and mixed type of inhibition respectively (Figs. 4a and 5a).

Determination of Michaelis–Menten Constant

The Michaelis–Menten constant (K_m) obtained from Lineweaver–Burk plots for native ChC was $K_m=0.55$ mM. This K_m value of the FALGPA hydrolysis by ChC is found to be similar to that obtained earlier [24]. The V_{\max} was found to be 0.2439 ± 0.05 mmol s^{-1} . From the Michaelis–Menten constants (Table 1), the addition of 160 μg of WSE and CHE to ChC resulted in K_m values of 3.33 ± 0.007 and 1.612 ± 0.002 mM, respectively. And then six lines obtained with only an enzyme and five concentrations of WSE and CHE intercept at the same and different positions, respectively, on the Y -axis, indicates that V_{\max} is the same for WSE and different for CHE in all cases (Table 1). Furthermore, the intercept of the lines on the X -axis demonstrates that the K_m value increases when the WSE and CHE concentration increases. These results further verify that the inhibition of ChC by WSE and CHE are the competitive and mixed type inhibitions.

Determination of the Inhibition Constant

The inhibition constant (K_i) was obtained from a Dixon plot of V^{-1} vs. various concentrations of WSE and FALPGA (Fig. 5b). For the determination of the $K_i=20$ of WSE against ChC activity, the velocity of cleavage of FALGPA was measured at WSE

concentrations ranging from 10 to 160 μg . In this plot, reciprocals of velocity were plotted against inhibitor concentration at different concentrations of FALGPA. A series of straight lines was obtained, converging at the same point, and that value represents $-K_i$. It was found that the K_i for WSE is 20 μg . The competitive mode of inhibition exhibited by WSE may work through direct competition with the substrate by binding to the active site, or binding upon a remote site and causing a conformational change in the ChC. Both the plots give identical positive results. This is important to study if there are any changes in the conformation of ChC on the binding of the WSE in the competitive type of inhibition.

Conformational Changes of WSE- and CHE-Treated ChCs

The effect of the WSE and CHE on the conformation of ChC has been investigated by monitoring circular dichroism (CD) spectral changes (Fig. 6a, b). In the far UV region, ChC exhibits double minima at about 210 and 220 nm and a maximum at 195 nm with a crossover point at about 200 nm. ChC has α helix, β sheet, β turn, and random coil structure. CD spectrum of the native ChC exhibits double-negative bands at 208 and 222 nm and a positive band around 195 nm. These results are characteristics of α helix, whereas a peptide/protein having β sheet conformation alone will exhibit a single negative band between 215 and 225 nm. ChC has both α helix and β sheet are almost equally distributed as its secondary structure, which may not be clearly distinguishable through the CD spectrum. The structure of ChC changes significantly to β sheet after interaction with WSE and CHE (80 μg), which are observed in the spectra where a negative band at 208 nm shifts to 215 nm, which is characteristic of β sheet (Fig. 6a). Treatment of low concentration (40 μg) of CHE changes the structure of ChC to β sheet, and with the further increase in concentration of CHE, the structure is shifted more towards the random coil conformation (Fig. 6b). Any structural alterations in an enzyme are known to affect the enzyme–substrate recognition process, as the structural fitting of enzyme in binding with the substrate is essential. The structural alterations in collagenase on interaction with the WSE and CHE were implicated in the inhibition of collagenolytic activity.

Discussion

The possibility to use plant extracts for MMP inhibition in degenerative diseases such as RA and OA has also been under research. There are lot of hydroxyl groups present in plant extracts that inhibit the collagenase activity, and then interaction with collagen backbone has been studied [30, 31]. The nature of interaction of the flavonoids, alkaloids, and polyphenolic with collagen and collagenase are currently under investigation [32]. While most of the current interest in the WSE and CHE relates to its antioxidant and anti-carcinogenic potential, the focus of the present work is on the potential of WSE and CHE to control the ChC activity against ECM degradation.

This investigation was undertaken to evaluate the inhibitory effect of WSE and CHE on the ChC activity in a dose-dependent manner. WSE and CHE exhibited a strong inhibitory effect against ChC activity. The inhibition of collagenase activity by green tea polyphenol has already been reported [33]. The increasing inhibition with increasing concentrations of WSE and CHE has been observed against ChC hydrolysis of collagen; this is indicative the effectiveness of the flavonoids, alkaloids, and withanolides in direct inhibition of ChC.

Inhibition kinetic parameters characterize different aspects of WSE- and CHE-treated ChC interaction with FALGPA. The inhibition of ChC occurs through binding of WSE and

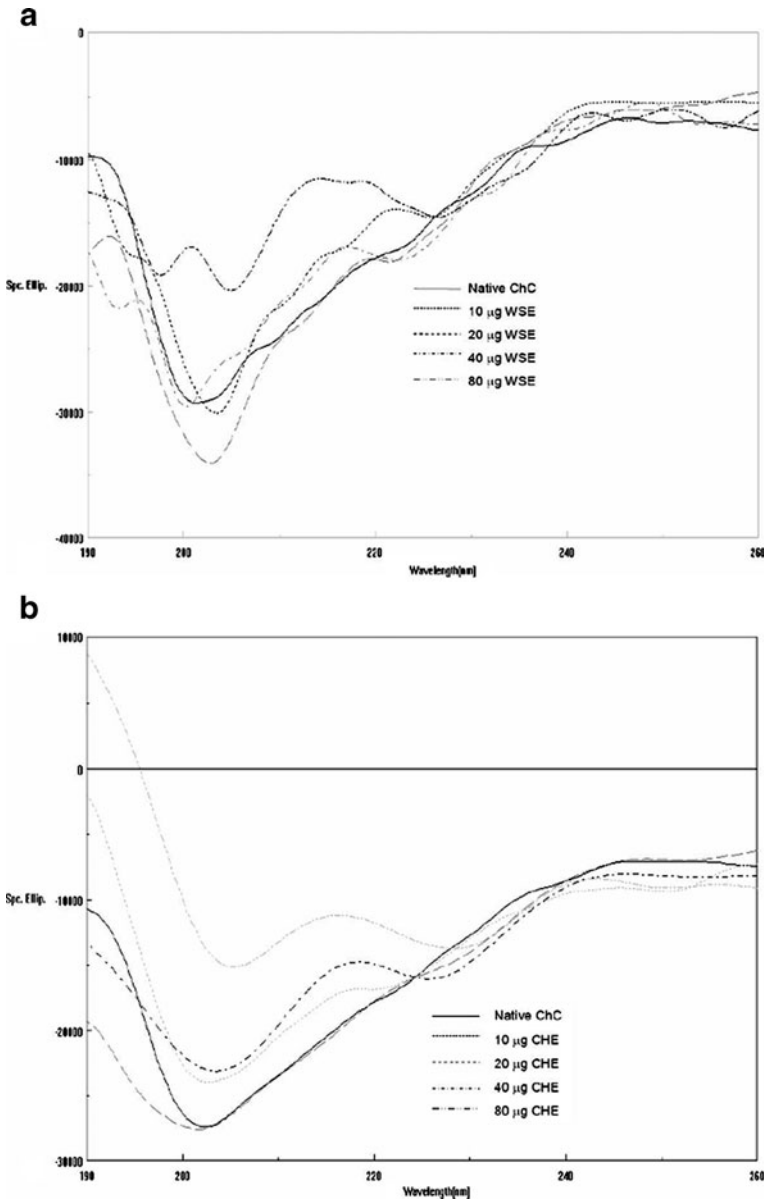
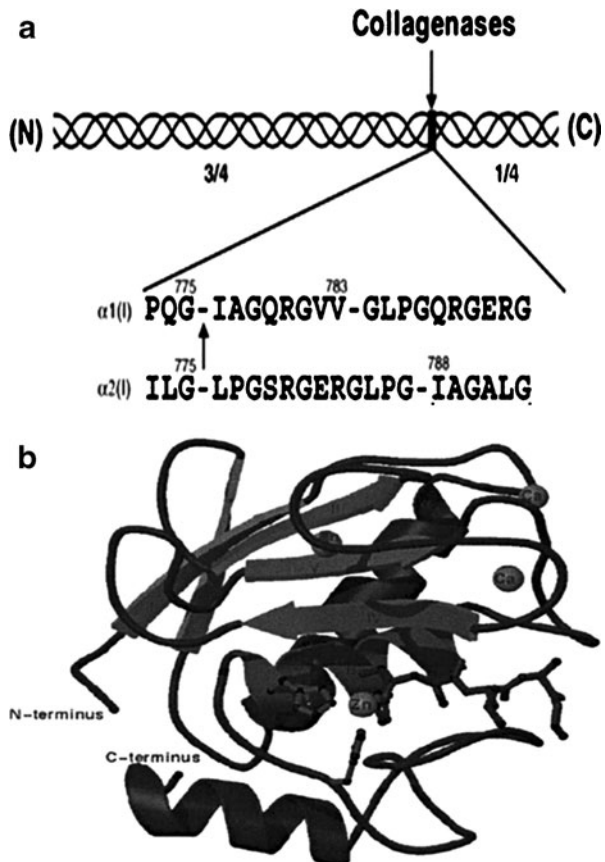


Fig. 6 CD spectral analysis of ChC (0.2 mg/ml in millimoles acetate buffer of pH 4.0 and at 25 °C) treated with different concentrations of **a** WSE and **b** CHE

CHE to the active part of the enzyme or inhibiting the activation. This kinetic study provides an insight into possible roles of WSE and CHE on the inhibition of ChC activity against hydrolysis of FALGPA. The ability of different concentrations of WSE and CHE to function as inhibitors is deduced from the values of V_{max} , K_m , and K_i obtained for the hydrolysis of FALGPA by ChC. WSE and CHE behave as competitive and mixed types of

inhibitors, respectively, against ChC activity. These MMPs-collagenases cleave the three α -chains of native triple-helical types I, II, and III collagens after Gly in a particular sequence (Gln/Leu)–Gly#(Ile/Leu)–(Ala/Leu) (# indicates the bond cleaved) located approximately three quarters away from the N terminus of the collagen molecule. The action of these enzymes is critical for the initiation of collagen breakdown, as once collagens are cleaved into three fourth and one fourth fragments they denature at body temperature and are degraded by gelatinases and other nonspecific tissue proteinases (Fig. 7a). The primary structures of the collagenases show that the proteins are similar and modular, each possessing a signal peptide of 20 residues, a propeptide domain (80 amino acids) conferring latency, a zinc-containing catalytic domain of approx. 180 residues, and a substrate specificity domain (210 amino acids). The three-dimensional scaffold of the catalytic domain is composed of a five-stranded β -sheet and three α -helices (Fig. 7b), similar to the β -sandwich architecture observed in zinc-dependent endopeptidases of this superfamily [34]. A deep cleft around the catalytic zinc located towards the C terminus of the domain delineates the active site- and substrate-binding regions of the protease. Three histidine residues of the protein and atoms of the bound inhibitor chelate the catalytic zinc. Additionally, structural zinc and several calcium atoms stabilize the structure of collagenase. Both the extracts were found to be the preferred chelators for the preparation of collagenase inhibitors. The observations of the present study on the behavior of WSE

Fig. 7 Cleavage sites in collagen I by **a** MMP-1 or ChC and **b** catalytic domain of ligand-free fibroblast collagenase (MMP-1) shown in a simplified form and calcium and zinc atoms are shown as pale spheres (34)



and CHE may be interpreted as follows. It has been shown experimentally that the side-chain carboxyl groups of aspartic (Asp) and glutamic acids (Glu) participate in conjunction with tyrosine (Tyr) residues in the stabilization of the active conformation of the native ChC. The competitive and mixed type ChC inhibition elicited by WSE and CHE, respectively, may arise from the binding of active components with side-chain carboxyl residues of aspartic (Asp) or glutamic acids (Glu), with the net result of change in the conformation of ChC. We found that WSE and CHE are competitive and mixed types of inhibitors for the ChC activity against FALGPA degradation, respectively. The structural changes of ChC after interaction with WSE and CHE observed. These results suggest that the steric structure of flavonoids, withanolides, and polyphenols containing steroidal lactone unit and hydroxyl group are important for the inhibition of ChC activity.

This study has provided convincing evidence of the protective effect of WSE and CHE against collagenolytic degradation of collagen. These results are clear that the change in the conformation of ChC by WSE and CHE are a major factor in the inhibition of collagenolytic activity. This may in part be a promising mechanism for the protection of collagen against collagenolytic activity. Therefore, both the extracts provide selective inhibition of ChC and could constitute an important aid in the prevention of arthritis as well as part of an effective therapy in the treatment of joint disease and other pathologies involving the action of collagenase. Further research to investigate the exact mechanism of action of WSE and CHE against arthritis is in progress.

Acknowledgment The first author acknowledges the Council of Scientific & Industrial Research (CSIR), New Delhi, Government of India, for his CSIR-SRF fellowship.

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